Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 18, Issue of May 3, pp. 15237–15240, 2002

Identification of a Neuronal Cdk5 Activator-binding Protein as Cdk5 Inhibitor*

Received for publication, January 16, 2002,
and in revised form, February 25, 2002
Published, JBC Papers in Press, March 6, 2002,
DOI 10.1074/jbc.C200032200

Yick-Pang Ching‡§, Andy S. H. Pang‡, Wing-Ho Lam‡, Robert Z. Qi¶‖, and Jerry H. Wang‡**

From the ‡Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China and the ¶Department of Medical Biochemistry, Faculty of Medicine, University of Calgary, Calgary, Alberta T2N 1N4, Canada

Neuronal Cdc2-like kinase (Nclk) plays an important role in a variety of cellular processes, including neuronal cell differentiation, apoptosis, neuron migration, and formation of neuromuscular junction. The active kinase consists of a catalytic subunit, Cdk5, and an essential regulatory subunit, neuronal Cdk5 activator (p35^{nck5a} or p25^{nck5a}), which is expressed primarily in neurons of central nervous tissue. In our previous study using the yeast two-hybrid screening method, three novel p35^{nck5a}-associated proteins were isolated. Here we show that one of these proteins, called C42, specifically inhibits the activation of Cdk5 by Nck5a. Co-immunoprecipitation data suggested that C42 and p35^{nck5a} could form a complex within cultured mammalian cells. Deletion analysis has mapped the inhibitory domain of C42 to a region of 135 amino acids, which is conserved in Pho81, a yeast protein that inhibits the yeast cyclin-dependent protein kinase Pho85. The Pho85·Pho80 kinase complex has been shown to be the yeast functional homologue of the mammalian Cdk5/p35^{nck5a} kinase.

Cyclin-dependent protein kinases (Cdks)[1] play critical roles in the regulation of cell division (1). As the name implies, functional Cdks require binding of a cyclin for kinase activity. In addition to depending on cyclin for activity, Cdk activities are regulated by complex mechanisms including protein phosphorylation and association with specific Cdk inhibitors. There are two Cdk inhibitor families: INK4 and Kip/Cip. Crystallography analysis has shown that the INK4 family member p16^{INK4a} causes the kinase inhibition by direct association to Cdk4 (4). On the other hand, p27$^{Kip/Cip}$ binds to both Cdk2 and cyclin A in the Cdk2·cyclin A complex to inhibit the kinase activity (5).

Cyclin-dependent kinase 5 is unique among Cdks in many respects. Unlike most other Cdks, Cdk5 has no known function in the cell division cycle but is involved in the regulation of neuronal differentiation and neurocytoskeleton dynamics (6–10). There are two mammalian Cdk5 activators: neuronal Cdk5 activator, p35^{nck5a}, and neuronal Cdk5 activator isoform, p39^{nck5ai}; both Nck5a and Nck5ai are expressed predominantly in neurons of central nervous systems (11, 12, 20). While Nck5a and Nck5ai are homologous proteins, they show little or no sequence similarity to cyclins. Members of both Cdk inhibitor families have been tested and shown to have no activity toward Cdk5. Biochemical analysis of Cdk5 in bovine brain extract suggests the existence of an inhibitory factor that exists together with Cdk5 and p35^{nck5a} in a protein complex (13). However, the molecular identity of the factor has not been established. In *Saccharomyces cerevisiae*, there is a cyclin-dependent protein kinase, Pho85, and its cyclin partner Pho80, which have been shown to be the functional homologues of mammalian Cdk5 and Nck5a, respectively (14). In addition, there is a specific Pho80/Pho85 inhibitor protein, Pho81, whose mammalian functional homologue is not known.

Recently we have used the yeast two-hybrid system to screen for the p35^{nck5a}-associated protein (15). Among the positive clones are three clones, called C42, C48, and C53, whose cDNA sequences are novel (16). In the present study, we show that one of the novel proteins, C42, displays an inhibitory effect on Cdk5 kinase activity. Interestingly, while both the full-length C42 and the C42 fragment obtained from the yeast two-hybrid screen can bind Nck5a, only the full-length protein has Cdk5 inhibitory activity. Using deletion mutants, we have mapped the inhibitory domain of C42 to a 135-amino acid region, which shows a significant homology to the inhibitory region of Pho81.

MATERIALS AND METHODS

Cloning and Construction of Plasmids—The DNA fragment encoding full-length C42 (C42-FL) and C42 binding fragment were subcloned into pCMV-Myc (CLONTECH) via *Eco*RI/*Not*I and *Xho*I/*Kpn*I sites, respectively. To generate the deletion mutants of C42, the pGEX-C42-FL plasmid was digested with *Nhe*I/*Not*I for the N56 mutant, *Sma*I for the N168 mutant, *Xba*I/*Not*I for the N279 mutant, *Sty*I/*Not*I for the N360 mutant, and *Hin*dIII/*Not*I for the N492 mutant, filled in at the adhesive end, and re-ligated. To make the FLAG-p35^{nck5a} expression construct, the DNA encoding p35^{nck5a} was retrieved by digestion with *Hin*dIII, filled in, and then cut with *Bam*HI. The *Bam*HI/blunt DNA fragment was ligated into pFLAG-CMV2 (Kodak) via *Bam*HI/*Sma*I sites.

Purification of the Proteins—The glutathione *S*-transferase (GST) fusion forms of C42, Cdk5, and Cdk2 proteins were purified according to a previous protocol (17). p25, p35, and p39 were subcloned into the pET32 vector, and His-tagged proteins were purified using the nickel-nitrilotriacetic acid beads (Invitrogen).

Cell Culture and Transfection—HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C in a humidified atmosphere at 5% CO_2. Transient transfection was carried out using LipofectAMINE Plus reagent (Invitrogen). Cells were incubated for 24 h before harvesting. The cells were washed twice in PBS and lysed with 100 µl of lysis buffer (50 mM Hepes, pH 7.2, 250 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 5 mM sodium fluoride, 10 µg/ml leupeptin, 1 µg/ml antipain, 2 mM phenylmethylsul-

* This work was supported by a grant from the Research Grants Council of Hong Kong and Hong Kong AoE of Molecular Neuroscience. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Current address: Inst. of Molecular Biology, Hong Kong University, Pokfulam, Hong Kong, China.

‖ Current address: Inst. of Molecular and Cell Biology, 30 Medical Dr., Singapore 117609, Singapore.

** To whom correspondence should be addressed. Tel.: 852-2358-8701; Fax: 852-2358-1552; E-mail: jerwang@ust.hk.

[1] The abbreviations used are: Cdk, cyclin-dependent protein kinase; Nck5a, neuronal cyclin-dependent protein kinase 5 activator; Nclk, neuronal Cdc2-like kinase; GST, glutathione *S*-transferase; FL, full length; BF, binding fragment; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; CKI, cyclin kinase inhibitor.

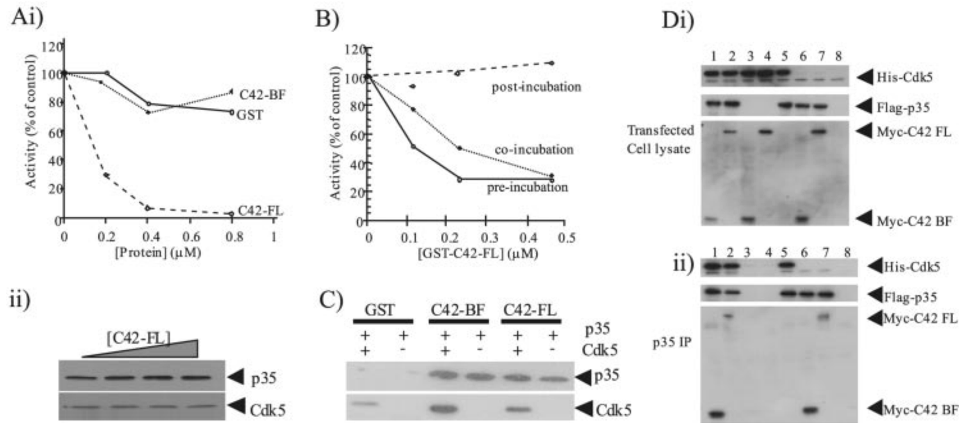

FIG. 1. **Inhibition of Cdk5 kinase activity by full-length C42 protein.** *Ai*, increasing amounts of full-length (*C42-FL*, *open diamond*) and binding fragment (*C42-BF*, *solid diamond*) of C42 proteins in GST fusion form were incubated with His$_6$-p35 (0.5 μg) and GST-Cdk5 (1 μg) for 30 min at 30 °C before being assayed for the histone H1 kinase activity as described under "Materials and Methods." GST (*open circle*) protein was used as a control. *Aii*, an aliquot (⅕) of the reaction mixture from C42-FL samples after incubation was loaded onto as SDS-polyacrylamide gel, transferred, and immunoblotted using anti-p35 antibody (C-19) and anti-Cdk5 antibody (C-8). *B*, increasing amounts of GST-C42-FL protein were incubated with His$_6$-p35 for 30 min followed by addition of GST-Cdk5 and reconstitution for another 30 min (*pre-incubation*). For co-incubation, all three proteins were mixed together at the same time, but for the postincubation samples, His$_6$-p35 and GST-Cdk5 were mixed up first before addition of GST-C42-FL. The samples were assayed for kinase activity after incubation. *C*, the GST-C42-BF, GST-C42-FL, and GST were preincubated with His$_6$-p35, washed with PBS, and divided into two portions. For one portion, GST-free Cdk5 was added (+), and the other portion remained unaffected (−). The protein complexes after incubation were precipitated by GSH-agarose beads, transferred, and immunoblotted with anti-Cdk5 and anti-p35 antibodies. *D*, co-immunoprecipitation of C42 and p35. *Di*, HeLa cells were co-transfected with constructs expressing His-Cdk5, FLAG-p35, Myc-C42-FL, and Myc-C42-BF. 10 μg of the transfected cell lysates were loaded onto each lane and immunoblotted with anti-Cdk5 (C-8), anti-FLAG (M2), and anti-Myc antibody (A-14). The combination of plasmids used were: *lane 1*, p35/Cdk5/C42-BF; *lane 2*, p35/Cdk5/C42-FL; *lane 3*, Cdk5/C42-BF; *lane 4*, Cdk5/C42-FL; *lane 5*, p35/Cdk5; *lane 6* p35/C42-BF; *lane 7*, p35/C42-FL, and *lane 8*, vector control. *Dii*, monoclonal anti-FLAG (M2) antibody was used to immunoprecipitate the p35 from the transfected cell lysates, and immunoprecipitants were loaded onto the SDS-polyacrylamide gel. Western blotting was performed using the specific polyclonal antibodies, *i.e.* anti-Cdk5 (C-8), anti-p35 (C-19), and anti-Myc (A-14) antibodies. The loading sequence of the samples was the same as in *A*.

fonyl fluoride, and 100 μg/ml benzamidine) for 15 min at 4 °C. Protein concentration was determined by the Bradford assay (Bio-Rad).

Affinity Binding Assay—The GST fusion form of C42 protein (20 μg) was incubated with His-tagged p35^{nck5a} (1 μg) for 30 min followed by addition of GST-free Cdk5 (1 μg) and incubation for another 30 min at 30 °C. After 20 μl (50% slurry) of GSH-agarose beads (Amersham Biosciences) were added to the mixture and kept on ice for 1 h, beads were washed four times with 1 ml of PBS. The bound proteins were released by addition of 2× SDS loading buffer.

In Vitro Kinase Assay—The Cdk5 kinase assay was performed according to the method described previously (18). Since the GST-C42 full-length preparation was contaminated with degraded forms, the molar concentration indicated was a calculated proportion of the GST-C42 protein band to the total protein used. For the protein substrate assay, the reactions were stopped by addition of 2× SDS loading buffer, and the proteins were resolved by 15% SDS-PAGE.

Immunoprecipitation and Western Blotting—For immunoprecipitation, antibodies were incubated with transfected cell lysate at 4 °C for 1 h before the addition of protein G-Sepharose beads. For immunoblotting, blots were visualized by enhanced chemiluminescence according to the manufacturer's procedure (Amersham Biosciences). Anti-Cdk5 (C-8), anti-c-Myc (A-14), and anti-p35 (C-19) antibodies were obtained from Santa Cruz Biotechnologies, and anti-FLAG (M2) antibody was obtained from Sigma.

RESULTS

Inhibition of Nck5a Activating Activity of Cdk5 Kinase by Full-length C42 Protein—The C42 clone obtained from the yeast two-hybrid screen encodes a polypeptide of 112 amino acid residues corresponding to Glu^{475}–Thr^{586} (the C terminus). This polypeptide displays high affinity Nck5a binding and is referred to as C42-BF. The C42-FL and C42-BF were bacterially expressed in GST fusion forms and tested for their effect on Cdk5 kinase activity. Fig. 1*Ai* shows that C42-FL inhibited Cdk5 kinase markedly in a dose-dependent manner, whereas the C42-BF or the GST protein has little effect on Cdk5 activity. The full-length form of the other two novel binding proteins, *i.e.* C48 and C53, were also tested and found to have no Cdk5 kinase inhibitory activity (data not shown). Western immunoblot analysis of the incubation reaction sample showed that quantities of Cdk5 and the activator remained unchanged after incubating with increasing amounts of C42-FL protein (Fig. 1*Aii*), suggesting that the loss of Cdk5 kinase activity could not be attributed to the possible contamination of proteases in the C42-FL sample.

The C42 inhibition of Cdk5 depends on the order of addition of C42 to the Cdk5 activation reaction. The kinase inhibition could be readily observed if C42 was incubated with p35^{nck5a} prior to the addition of Cdk5 or if the three proteins, C42, p35^{nck5a}, and Cdk5, were mixed simultaneously. On the other hand, preincubation of Cdk5 with p35^{nck5a} markedly lowered the inhibitory effect of C42 (Fig. 1*B*). To examine whether p35^{nck5a} could still form a complex with Cdk5 after binding with C42 protein, affinity pull-down experiments were performed. A sample of p35^{nck5a} preincubated with the GST-fused full-length C42 or C42 fragment was tested for the ability to associate with Cdk5. As shown in Fig. 1*C*, both GST-C42-FL·p35^{nck5a} and GST-C42-BF·p35^{nck5a} preformed complexes were able to pull down Cdk5. In some experiments, a small amount of Cdk5 was found to co-precipitate with the GST control. The reason for this is not clear. In contrast to p35^{nck5a}-bound GST-C42, free GST-C42, either the full-length or the fragment, did not pull down Cdk5 in the affinity binding assay (data not shown). The observation that the full-length C42·p35^{nck5a} complex can bind Cdk5 indicates that C42 inhibition is not due to a competition between C42 and Cdk5 for p35^{nck5a} binding.

To test whether or not C42 protein could associate with Nck5a within mammalian cells, co-immunoprecipitation experiments were carried out. The full-length C42 or the C42 fragment was transfected into HeLa cells and expressed as Myc-tagged protein together with FLAG-tagged p35^{nck5a} and His-tagged Cdk5. Transfected cell lysates, which contained similar amounts of the expressed proteins, were selected for the co-immunoprecipitation experiment (Fig. 1*Di*). As shown in Fig. 1*Dii*, the Myc-C42 (either the full-length protein or C42 fragment) and Cdk5 were found in the immunoprecipitates from the triple transfected cell lysates, *i.e.* lysates from cells transfected with p35^{nck5a}/C42/Cdk5, in the anti-p35 antibody-mediated precipitation. On the other hand, the immunoprecipitates from the control cell lysates, *i.e.* lysates from cells transfected with C42/Cdk5 and p35^{nck5a}/Cdk5, did not contain Myc-C42. These results suggest that p35^{nck5a} can form a complex with C42 and Cdk5 inside the cells. However, attempts to use anti-Myc antibody for immunoprecipitation were not successful because the antibody did not precipitate the Myc-tagged C42 protein effectively even when an excessive amount of the antibody was used (data not shown).

Specificity of the Inhibition of p35^{nck5a}/Cdk5 by C42—A series of experiments were carried out to test the specificity of the kinase inhibition activity of C42. Cyclin A-activated Cdk2 has catalytic properties similar to p35^{nck5a}/Cdk5 kinase. Fig. 2*A* shows that although co-incubation of the full-length C42 with Cdk5 and p35^{nck5a} abolished almost 90% of the kinase activity, co-incubation of Cdk2 and cyclin A with C42 protein had little or no effect on Cdk2 activity. Nck5a exists in the brain or cell extracts in a 35-kDa or a 25-kDa form, and both forms of the protein associate with Cdk5 (11, 12, 19). Furthermore, mammalian brains contain an isoform of Nck5a, p39^{nck5ai}, that also associates with and activates Cdk5 (20). To further test the C42 inhibition specificity, the effects of C42 on Cdk5 activated by these various forms of activator proteins were examined. As shown in Fig. 2*B*, C42 is capable of inhibiting Cdk5 irrespective of the form of the activator protein used. An early study had shown that Nck5a, in addition to activating Cdk5, can also activate Cdk2 albeit to an activity level much lower than that achieved by cyclin A (17). Fig. 2*C* shows that although cyclin A-activated Cdk2 was refractory to C42 inhibition, p35^{nck5a}-activated Cdk2 was efficiently inhibited by the full-length C42. In addition to demonstrating the specificity of C42 inhibition toward p35^{nck5a}, these results rule out the possibility that the loss of kinase reaction is attributed to the existence of protease, ATPase, or phosphatase activity in the C42 sample.

Identification of the N-terminal Inhibitory Region of C42—To map the inhibitory domain of C42, a series of C-terminal deletion mutants were generated, including N492, N360, N168, and N56 (the number refer to the residues left from the N-terminal). All of the mutants were able to inhibit the p35^{nck5a}/Cdk5 kinase activity except N56 (Fig. 3*A*) (data not shown for the N492 mutant). The result indicates that the Cdk5 inhibitory domain is contained in the N-terminal region (residues 1–168) of the C42 protein. This region does not contain the sequence corresponding to C42-BF, thus suggesting the existence of separate Nck5a-binding and Cdk5 inhibition domains in C42. *S. cerevisiae* protein Pho81 displays specific inhibitory activity toward the yeast Cdk5/Nck5a homologue, *i.e.* Pho85/Pho80. Alignment of C42 and Pho81 has revealed a moderate sequence homology (19% identity and 56.8% similarity). Recently the inhibitory domain of Pho81 has been identified to comprise about 80 residues, residues 645–724 (21). When this domain was compared with the full-length C42 protein sequence, it aligned to the N-terminal region of C42 from residues 22–157 (Fig. 3*B*). Note that this is within the N-terminal region of C42 (residues 1–168) expected to contain the Cdk5 inhibitory domain. To further test whether the region comprising residues 22–157 of C42 is the Cdk5 inhibitory domain, this portion of C42 was expressed bacterially and tested for Cdk5 inhibitory activity. As shown in Fig. 3, *C* and *D*, the GST fusion form of the inhibitory domain can suppress the phosphorylation of both protein and peptide substrates by Cdk5 kinase. In the assay using histone H1

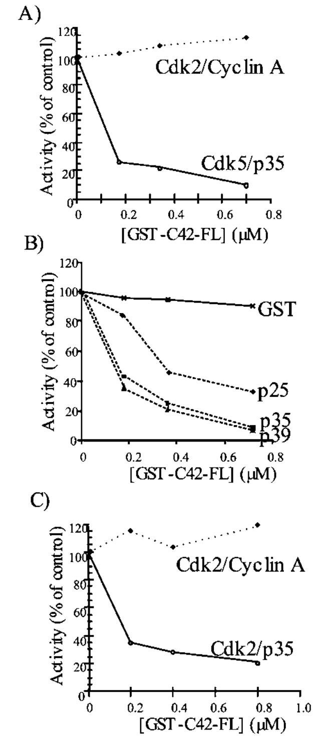

FIG. 2. **Specific inhibition of Nck5a by C42 protein.** *A*, GST-Cdk2 (1 μg)/His$_6$-cyclin A (0.5 μg) and GST-Cdk5 (1 μg)/His$_6$-p35 (0.5 μg) were reconstituted in the presence of increasing concentrations of full-length GST-C42 protein for 30 min at 30 °C and then assayed for the histone H1 kinase activity. The activity was expressed as percentage of control. *B*, proteins were reconstituted similar to those in *A*, but His$_6$-p35 (0.5 μg), His$_6$-p25 (0.5 μg), and His$_6$-p39 (0.5 μg) were used to activate GST-Cdk5. For GST control, the GST-C42 was replaced by an equal amount of GST protein in the assay of His$_6$-p39·GST-Cdk5 complex. *C*, proteins were reconstituted similar to those in *A*, but GST-Cdk2, instead of GST-Cdk5, was used for reconstitution with His$_6$-p35.

protein as substrate, addition of C42 inhibitory domain had reduced about 60% of the Cdk5 kinase activity (Fig. 3*D*).

DISCUSSION

Previously we have used the yeast two-hybrid screen to isolate three novel p35-binding proteins, named C42, C48, and C53, and showed that they bind to p35^{nck5a}·Cdk5 complex (16). In this study, we demonstrate that the full-length C42, but not the C42 fragment from the yeast two-hybrid screen, possesses potent inhibitory activity toward Cdk5. We have carefully ruled out a number of artifacts that may give rise to an apparent kinase inhibition, such as the contamination of protease, ATPase, or phosphatase activity in the C42 sample. Affinity precipitation using bacterially expressed proteins indicates that either full-length C42 or C42 fragment can form a ternary complex with Cdk5 and Nck5a. Immunoprecipitation analysis shows that the ternary complexes can also exist in HeLa cell lysates. These results strongly suggest that C42 is a Cdk5 inhibitor. In fact, we have carried out transfection experiments showing that ectopic expression of the full-length C42 in NG108 neuroblastoma cells markedly suppressed the differentiation of the cells in differentiating medium, whereas the expression of C42-BF had significantly less effect.[2]

The amino acid sequence of C42 shows no sequence similarity to proteins of either CKI family. The mechanism of Cdk5 kinase inhibition of C42 also appears to distinguish the protein from the known CKIs. While CKIs of the Kip/Cip family interact through the kinase catalytic subunit, members of the INK4

[2] Y.-P. Ching, W.-H. Lam, and J. H. Wang, unpublished observation.

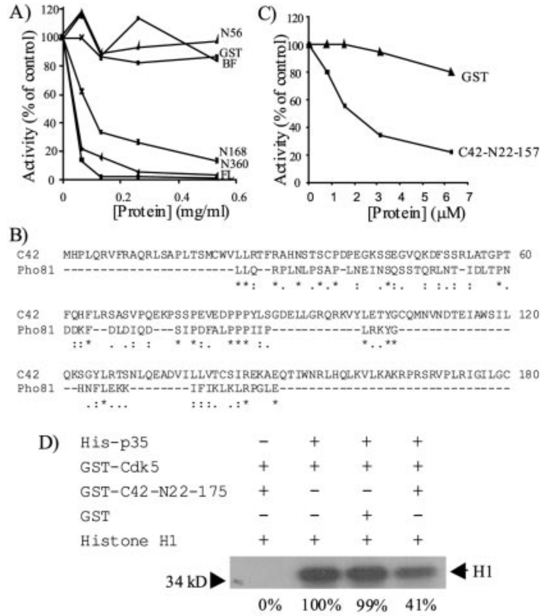

FIG. 3. **Mapping of the inhibitory region of C42.** *A*, deletion mutants of C42 were assayed for the inhibition of p35·Cdk5 kinase complex. Different concentrations of the full length (*FL*), binding fragment (*BF*), GST control, and the mutants including N360, N168, and N56 were reconstituted similar to proteins in Fig. 2*A*, and the kinase activity was assayed as described. *B*, the inhibitory domain of Pho81 was aligned with the full-length sequence of C42 by the ClustalW program. The *numbers* at the *right-hand side* of the C42 sequence represent the position of the residue corresponding to the full-length sequence. *Asterisks* indicate identical residues, and *semicolons* indicate similar residues. *C*, the fragment C42-N22–157 was tested for the inhibition of p35·Cdk5 kinase complex. Different amounts of the protein were incubated with the kinase complex, and the activity was measured using histone H1 peptides. *D*, the inhibition of Cdk5 kinase activity by the C42-N22–157 fragment was assayed using histone H1 protein substrate as described under "Materials and Methods." The phosphorylation of histone H1 proteins was quantitated by densitometry, and the relative intensity of the signals in percentage was indicated at the *bottom* of the band.

family undergo physical interactions with both the kinase and the cyclin subunits. In contrast, C42 appears to inhibit Cdk5 activity by interacting exclusively with the regulatory subunit Nck5a (or Nck5ai). Affinity binding experiments have shown that C42 undergoes high affinity binding to both Nck5a (or Nck5ai) and the Nck5a·Cdk5 complex but not to monomeric Cdk5. The inhibitory activity of C42 shows a strict specificity toward Nck5a or Nck5ai rather than the kinase subunit. This may be demonstrated by using Cdk2 instead of Cdk5 as the inhibition target. Only the $p35^{nck5a}$-activated Cdk2 but not the cyclin A-activated Cdk2 can be inhibited by C42.

While there is a good correlation between C42 inhibition activity and the ability of the protein to undergo high affinity binding to Cdk5 activator proteins, the inhibition activity is not solely due to the protein binding. Both full-length C42 and the C42 fragment bind to Nck5a with high affinity, but only the full-length C42 displays kinase inhibitory activity. Despite the fact that the other two novel Nck5a-binding proteins, C48 and C53, share the same Nck5a binding region of C42, they did not exhibit Nck5a inhibitory activity. These observations have led us to speculate that the full-length C42 contains a kinase inhibitory domain in addition to the high affinity Nck5a-binding domain. Deletion analysis has suggested the existence of a kinase inhibitory domain distinct from the Nck5a-binding domain of C42, a suggestion subsequently confirmed from sequence comparison between C42 and the recently identified Cdk inhibition domain in Pho81 (see below). A simple model to account for the existence of both Nck5a-binding and kinase inhibition domains is that while kinase inhibition depends solely on the kinase inhibitory domain, C42 uses the high affinity Nck5a-binding domain to increase the affinity of the inhibitory domain-enzyme interaction. The fact that the inhibitory domain can only inhibit about 60% of the kinase activity may suggest that the Nck5a-binding domain is required for achieving high inhibitory activity. This is also in agreement with the observation that the concentration of the inhibitory domain required for kinase inhibition is about an order of magnitude higher than that of full-length C42.

A number of observations suggest that C42 is related to Pho81, a Cdk inhibitor that is specific for Pho85/Pho80, the yeast homologue of Cdk5/Nck5a. The kinase inhibitor domain of Pho81 has recently been mapped (21). Sequence alignment of C42 and Pho81 has identified a region in C42 showing significant similarity to the kinase inhibitory domain of Pho81. The C42 deletion mutant containing only this region of the protein displayed Cdk5 inhibitory activity. In addition to structural similarity, C42 and Pho81 are similar in their Cdk inhibition mechanisms. Results of complementary assays in yeast suggested that Cdk5 could associate with Pho80 to form an active kinase and that the active kinase responded to Pho81 in yeast growing in low phosphate medium. Like C42, Pho81 exerts its kinase inhibitory activity through interaction with the regulatory subunit Pho80, rather than with Pho85, the catalytic (Cdk) subunit. The kinase inhibition by C42 is strongly dependent on the order of addition of Cdk5 to Nck5a and Cdk5 (Fig. 1*B*). Intriguingly a ternary complex of Cdk5, Nck5a, and C42 can be formed under either the inhibiting or the noninhibiting conditions. Based on their functional analysis of Pho81 and Pho85/Pho80 interaction in yeast cells, Huang *et al.* (21) suggested that Pho81 might form either an active or an inactive ternary complex with Pho85/Pho80 kinase in high and low phosphate medium. Thus, from these various lines of evidence, it is suggested that C42 and Pho81 may be grouped into a new family of CKIs.

REFERENCES

1. Morgan, D. O. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 261–291
2. Deleted in proof
3. Deleted in proof
4. Jeffrey, P. D., Tong, L., and Pavletich, N. P. (2000) *Genes Dev.* **14,** 3115–3125
5. Pavletich, N. P. (1999) *J Mol. Biol.* **287,** 821–829
6. Dhavan, R., and Tsai, L. H. (2001) *Nat. Rev. Mol. Cell. Biol.* **10,** 749–759
7. Lew, J., and Wang, J. H. (1995) *Trends Biochem. Sci.* **20,** 33–37
8. Deleted in proof
9. Grant, P., Sharma, P., and Pant, H. C. (2001) *Eur. J. Biochem.* **268,** 1534–1546
10. Deleted in proof
11. Lew, J., Huang, Q. Q., Qi, Z., Winkfein, R. J., Aebersold, R., Hunt, T., and Wang, J. H. (1994) *Nature* **371,** 423–426
12. Tsai, L. H., Delalle, I., Caviness, V. S., Jr., Chae, T., and Harlow, E. (1994) *Nature* **371,** 419–423
13. Lee, K. Y., Rosales, J. L., Tang, D., and Wang, J. H. (1996) *J. Biol. Chem.* **271,** 1538–1543
14. Huang, D., Patrick, G., Moffat, J., Tsai, L. H., and Andrews, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14445–14450
15. Qi, Z., Tang, D., Zhu, X., Fujita Donald, J., and Wang, J. H. (1998) *J. Biol. Chem.* **273,** 2329–2335
16. Ching, Y. P., Qi, Z., and Wang, J. H. (2000) *Gene* (*Amst.*) **242,** 285–294
17. Tang, D., Chun, A. C. S., Zhang, M., and Wang, J. H. (1997) *J. Biol. Chem.* **272,** 12318–12327
18. Lew, J., Beaudette, K., Litwin, C. M., and Wang, J. H. (1992) *J. Biol. Chem.* **267,** 13383–13390
19. Qi, Z., Huang, Q. Q., Lee, K. Y., Lew, J., and Wang, J. H. (1995) *J. Biol. Chem.* **270,** 10847–10854
20. Tang, D., Yeung, J., Lee, K. Y., Matsushita, M., Matsui, H., Tomizawa, K., Hatase, O., and Wang, J. H. (1995) *J. Biol. Chem.* **270,** 26897–26903
21. Huang, S., Jeffery, D. A., Anthony, M. D., and O'Shea, E. K. (2001) *Mol. Cell. Biol.* **21,** 6695–6705
22. Deleted in proof
23. Deleted in proof